Detection of iron in tissues from slugs (*Deroceras reticulatum* Müller) after ingestion of iron chelates, by means of energy-filtering transmission electron microscopy (EFTEM)

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Abstract: Two iron chelates, one toxic (iron 'butan') and the other not (iron 'octan'), were ingested by slugs (*Deroceras reticulatum*) at either a low (5 g kg^{-1} diet) or a high (100 g kg^{-1} diet) dose rate. In tissue sections of the digestive gland and body wall, iron was detected by energy-filtering transmission electron microscopy (EFTEM), using electron spectroscopical imaging (ESI) and electron energy-loss spectroscopy (EELS). The strongest signals for iron were obtained in secondary lysosomes of the resorptive cells in the digestive gland of slugs treated with a low dose of either compound, or with the high dose of iron 'octan'. At the cell apices of these cells, in endocytotic vesicles and in apically located lysosomes, iron was detected only in slugs fed with either dose of iron 'octan'. In slugs fed with the high dose of iron 'butan', iron could clearly be localised in the epithelial and mucus cells of the skin. The results are discussed with respect to differences in the toxicity of the two iron chelates.

Keywords: *Deroceras reticulatum*; molluscicide; iron chelate; ESI; EELS

1 INTRODUCTION

In many parts of the world, molluscs cause economic damage in agriculture and horticulture. The slugs *Deroceras reticulatum* (Müller), *D. laeve* (Müller), and various *Arion* species, for example, damage forage legumes, soybeans, root crops and cereals.[1,2] During the last decade, molluscs have become increasingly important pests in aquatic systems, for example apple snails (*Pomacea canaliculata*) in rice fields,[3–5] pond snails (*Cerithium sp*) as consumers of algal food in fish-rearing ponds,[6] and biofouling molluscs such as *Dreissena polymorpha* Pall.[7] Molluscs' impact on man is not confined to their role as pests; for example some freshwater gastropod species of the genera *Biomphalaria* and *Bulinus* are intermediate hosts of the parasite *Schistosoma* and are therefore directly involved in transmission of the human disease bilharzia.[8]

Several different types of molluscicide are in use in terrestrial and aquatic ecosystems to control mollusc pests and mollusc-transmitted diseases[9–11] but there is still a need for new ones to improve efficacy, specificity and cost-effectiveness. Potent naturally occurring molluscicides such as saponins or tree extracts have been studied during recent years as agents to control aquatic gastropod species,[9,12,13] and a new class of molluscicide to control terrestrial slug pests, the metal chelates, has been introduced by Henderson *et al.*[14] Although data are available concerning metal chelates' toxicity compared to current products such as metaldehyde or methiocarb,[15–17] their mode of action is still unknown. It is also still unclear why the toxicity of iron chelates varies with the carbon chain length of the ligand. For example, in the homologous series based on tris(1-oxo-1,2-diazapropan-2-oxido)Fe(III), toxicity increases to a maximum at the 'diazapentan' stage. It is also known that there are differences in the distribution throughout a slug's body of iron from a toxic member of the series compared to that from a non-toxic homologue.[18] We have now used energy-filtering transmission electron microscope methods to study the

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Contract/grant sponsor: Biotechnology and Biological Sciences Research Council
(*Received 27 April 1998; revised version received 29 June 1998; accepted 3 August 1998*)

 Pestic Sci 0031-613X/99/$17.50

intracellular distribution of iron in slugs fed with iron chelates under the same experimental conditions. Relating the physiological functions of intracellular components to the sites where the iron was localised could contribute to the further elucidation of the mode of action of iron chelate molluscicides.

2 MATERIALS AND METHODS

Field slugs, *Deroceras reticulatum* (Müller), were collected and maintained in a controlled environment (12 h 15°C day/12 h 5°C night) on Chinese cabbage (*Brassica chinensis* Juslen). Healthy individuals weighing 400–600 mg were selected for tests as required and starved (24 h) before treatment. Each was fed with 5 mg of blended dry bait mixture (oven-dried, milled untreated wheat cv. Avalon + paraffin wax + sucrose; 87.5 + 10 + 2.5 by weight) containing either 25 µg or 500 µg per animal of either tris(1-oxo-1,2-diazabutan-2-oxido)Fe(III) or tris(1-oxo-1,2-diazaoctan-2-oxido)Fe(III), hereafter known respectively as iron butan and iron octan. Control slugs were fed bait mixture alone. The slugs were placed in the controlled-environment cabinet 3 h before scotophase. Four slugs per treatment, each of which had eaten all the bait, were removed, dissected and fixed 1 h after the start of the next photophase, 16 h after feeding had begun. Samples of digestive gland and body wall were fixed in 2% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.4) (2 h at 4°C), then postfixed in 1% aqueous OsO_4 (2 h at 4°C), dehydrated in a graded series of ethanol, and finally embedded in Spurr's resin.[19] Ultrathin sections of 30–40 nm were examined for iron in a Zeiss CEM 902 transmission electron microscope without further counterstaining.

For iron analyses, the sections were examined with an energy-filtering transmission electron microscope, CEM 902 (Castaing-Henry-Spectrometer, Zeiss, Oberkochen, Germany), equipped with a photomultiplier for recording electron energy-loss spectra, a highly sensitive SIT-TV-camera (Dage-MTI, Michigan City, IN, USA) and an integrated digital image-analysing system (Zeiss-Kontron, Oberkochen, Germany).

For elemental mapping of iron by electron spectroscopical imaging (ESI), the two-windows method was applied.[20] Images were recorded with the TV camera at 690 eV and 716 eV (below and above the iron-specific edge) with an energy-selective slit width of 8 eV each. Subtraction of the background image taken at 690 eV from the 716 eV image using digital image analysis displays the net distribution. The image is combined with the inverse picture of the structure-sensitive image taken at 250 eV and overlain by a false-colour table indicating the presence of iron by coloured spots on the test tissue. In black-and-white reproductions of the glossy prints, the coloured dots appear white on a dark background and black on a light background. To show that the signals in the electron spectroscopical images arose from the presence of iron in the tissue and not from other effects related to mass thickness, ghost images were taken from samples of skin and digestive gland at 690 eV with background images at 680 eV. Electron energy-loss spectra (EELS) were recorded from 620 to 780 eV with a slit width of 1 eV. The iron signal was located at 716 eV. In the skin samples, the intracellular distribution of iron was studied in mucocytes (mucus vacuoles, Golgi vesicles) and epithelial cells (apices with microvilli and vesicles), and, in the digestive gland, in resorptive cells (cell apices with microvilli, pinocytotic vesicles and primary lysosomes, secondary lysosomes in the cell centre) and basophilic cells (vacuoles and endoplasmic reticulum at the cell bases).

3 RESULTS

The results are summarised in Table 1.

3.1 Controls

A small amount of iron was detected in the large vacuoles which correspond to secondary lysosomes of the digestive gland. Small iron particles were dispersed within these vacuoles (Fig 1).

3.2 Iron butan, low dose

A clear signal for iron was obtained in the vacuoles of the mucocytes in the skin. In the digestive gland, clear signals for iron were found in secondary lysosomes of the resorptive cells, where the iron was found to be attached to highly electron-dense contents of the vacuoles (Figs 2 and 3). EELS spectra taken at these sites proved the signals obtained by ESI to represent iron with a peak at 716 eV (Fig 13). In the basophilic cells, iron was found in the cytoplasm, in small vesicles, in vacuoles characterised by electron-dense contents, and attached to the endoplasmic reticulum (Fig 4).

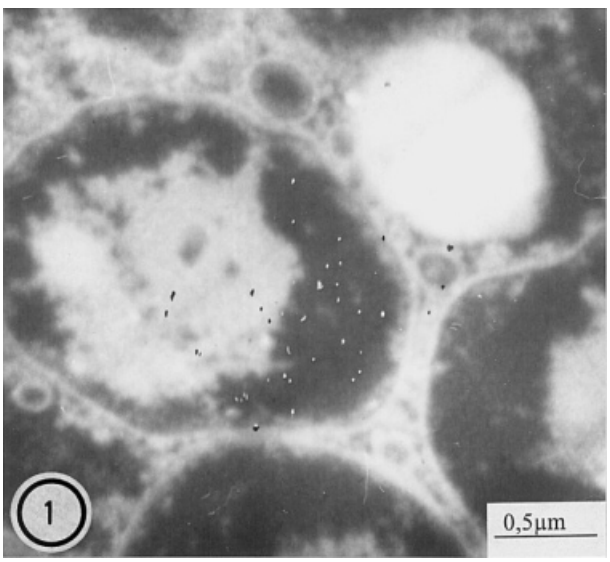

Figure 1. Control. Small iron particles in a secondary lysosome of the digestive gland.

Table 1. Distribution of iron in skin and digestive gland from slugs treated with iron butan and iron octan

Treatment	*Skin*			*Digestive gland*				
	Median % of total iron recovered (min–max)[a]	*Intracellular distribution*[b]		*Median % of total iron recovered (min–max)*[a]	*Intracellular distribution,*[b] *resorptive cells*		*Intracellular distribution,*[b] *basophilic cells*	
Control	–	a/mv	–	–	a/mv/p	–	er	–
		muv	–		v/l	–	v	–
		v	–		sl*d*	±		
Iron butan, low dose	2 (0–6)	a/mv	–	77 (52–84)	a/mv/p	–	er	+
		muv	+		v/l	–	v	±
		v	–		sl*f*	+ +		
Iron butan, high dose	24 (9–32)	a/mv	+	50 (29–70)	a/mv/p	–	er	±
		muv	+		v/l	–	v	–
		v	+ +		sl*d*	±		
Iron octan, low dose	1 (0–2)	a/mv	–	88 (61–90)	a/mv/p	±	v	+
		muv	–		v/l	±		
		v	–		sl*f*, *d*	+		
Iron octan, high-dose	0	a/mv	–	84 (60–89)	a/mv/p	±	er	–
		muv	–		v/l	±	v	±
		v	–		sl*f*, *d*	+		

[a] Results from Clark *et al*[18]: distribution of iron between different organs of slugs following ingestion of iron butan and iron octan under the same conditions.
[b] a: apical cytoplasm; er: endoplasmic reticulum; l: lysosome; muv: mucus vacuoles; mv: microvilli; p: pinocytotic vesicle; sl: secondary lysosome; v: vesicles/small vacuoles; *d*: dispersed, flocculent; *f*: focused, larger aggregates; – not detected; ± few iron particles detected; + clear iron detection; + + very strong iron signal.

3.3 Iron butan, high dose

Strong iron signals were found in the mucocytes and epithelial cells of the skin. The iron was found to be located in small and large mucus vacuoles (Figs 5 and 6) and in the cytoplasm (partly attached to electron-dense particles) of adjacent epithelial cells (Fig 6). Clear iron signals were also obtained in the extracellular space between microvilli of the epithelial cells of the skin, and in their apical cytoplasm and apical vesicles or vacuoles (Fig 7). In the digestive gland, very small iron particles were found dispersed within the vacuoles of the secondary lysosomes in the resorptive cells (Fig 8).

3.4 Iron octan, low dose

Iron was detected only in the digestive gland. Small iron particles were found in the lumen of the digestive gland tubules between the microvilli of the resorptive cells. Iron was also found in the cytoplasm of these cells and in small vesicles. In secondary

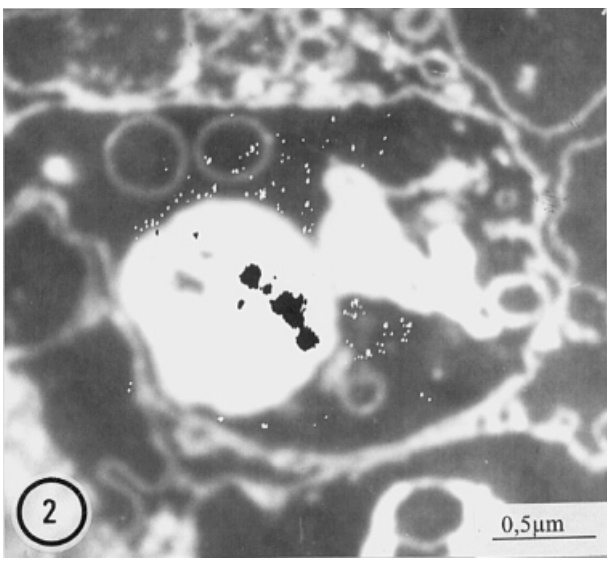

Figure 2. Iron butan, low dose. Iron attached to highly electron-dense contents of a secondary lysosome in a resorptive cell of the digestive gland.

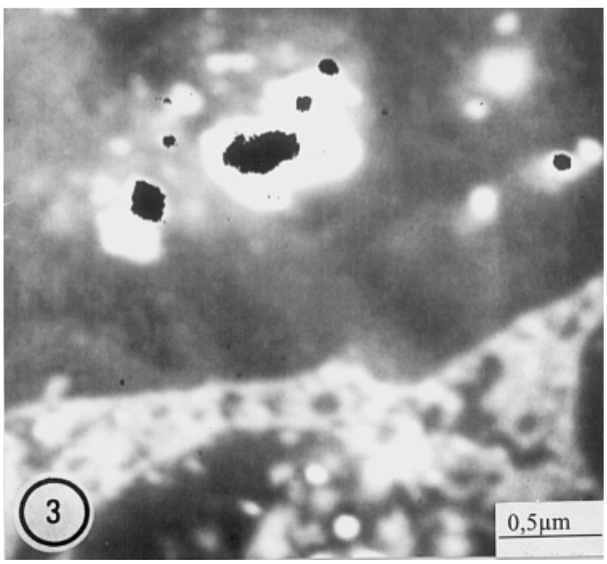

Figure 3. Iron butan, low dose. Iron attached to highly electron-dense contents of a secondary lysosome in a resorptive cell of the digestive gland.

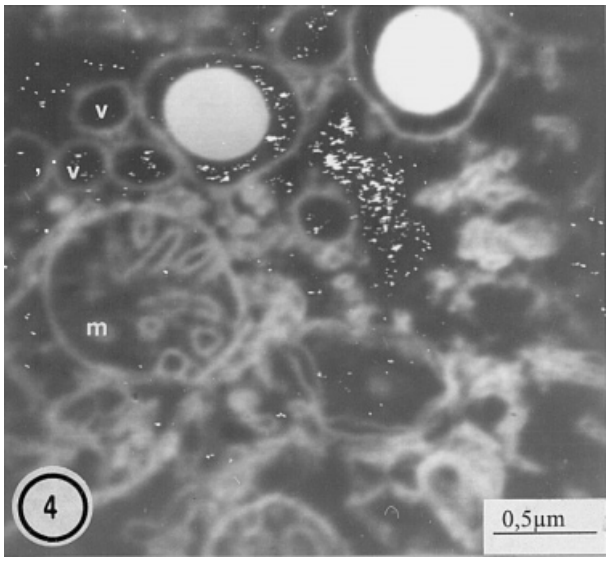

Figure 4. Iron butan, low dose. Iron in the cytoplasm, in vesicles (**v**) and in vacuoles characterized by electron-dense contents of a basophilic cell in the digestive gland. **m**: mitochondrion.

lysosomes, clear iron signals were obtained for electron-dense contents of these vacuoles; and small, dispersed iron particles were found surrounding these electron-dense contents (Fig 9). In the basophilic cells, iron was located between the cisternae of the endoplasmic reticulum as well as in small vacuoles (Fig 10).

3.5 Iron octan, high dose

No iron was found in the skin of slugs treated with the high dose of iron octan. A small amount of iron was found in the lumen of the digestive gland tubules between the microvilli of the resorptive cells, and also distributed throughout their apical cytoplasm and within vacuoles (Fig 11). In secondary lysosomes, clear iron signals were obtained from and around the electron-dense contents of the vacuoles (Fig 12). In the basophilic cells, the signals were

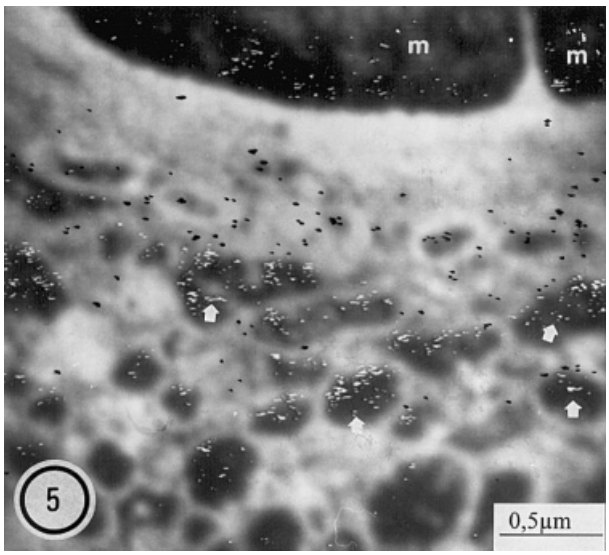

Figure 5. Iron butan, high dose. Iron signals in small (arrows) and large mucus vacuoles (**m**) in a mucocyte of the skin.

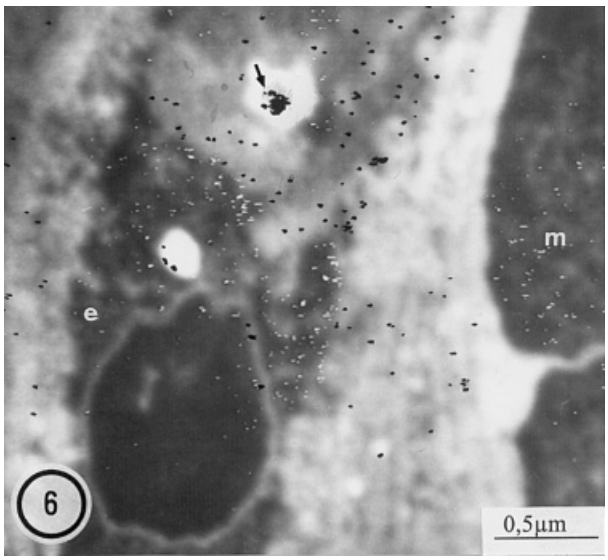

Figure 6. Iron butan, high dose. Iron signals in large mucus vacuoles of a mucocyte (**m**) and in the cytoplasm of an epithelial cell (**e**) of the skin, where it is located in the cytoplasm and attached to electron-dense particles (arrow).

less intense in animals which received the high dose than in those fed the low dose.

3.6 Ghost images

Ghost images taken between 680 eV and 690 eV in a secondary lysosome of the digestive gland and in a mucocyte of the skin did not show any signal. This confirmed that the signals obtained between 690 eV and 716 eV arose from the presence of iron in the tissue and not from effects related to mass thickness.

4 DISCUSSION AND CONCLUSIONS

In this study, the intracellular location of iron in skin and digestive gland tissue of slugs which had been

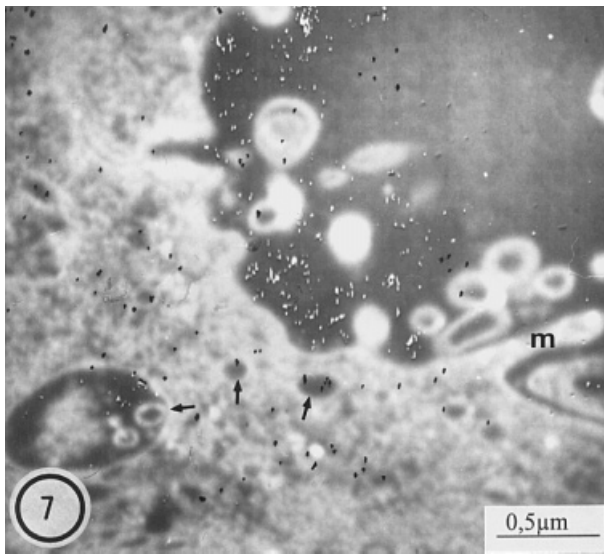

Figure 7. Iron butan, high dose. Iron signals at the apex of an epithelial cell of the skin, in the extracellular space between microvilli (**m**) and in the cytoplasm in apically located vesicles or vacuoles (arrows).

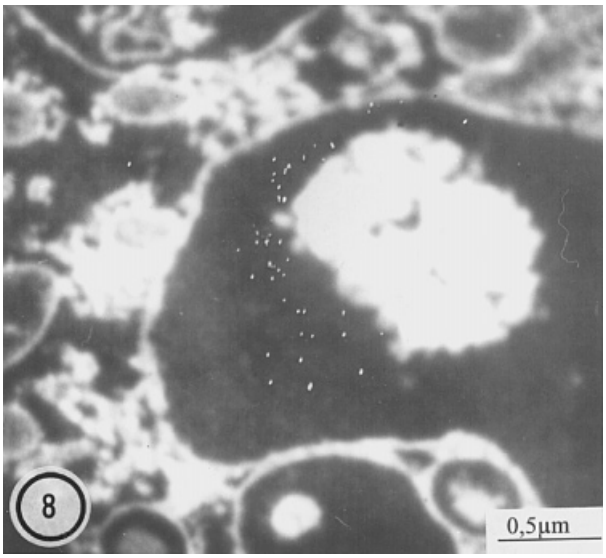

Figure 8. Iron butan, high dose. Small iron particles dispersed within the vacuoles of the secondary lysosomes in the resorptive cells of the digestive gland.

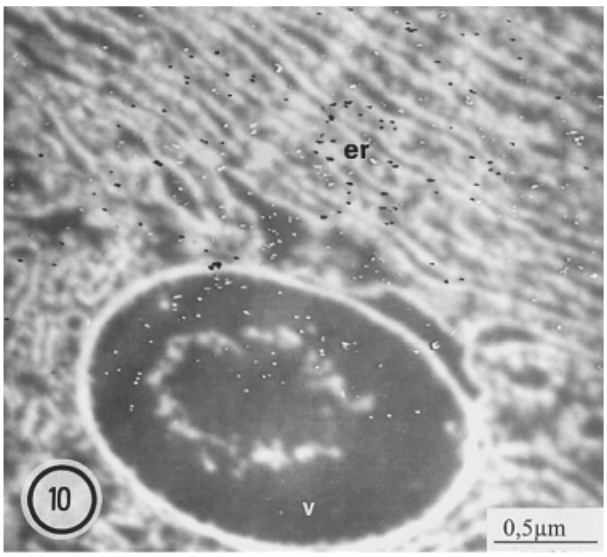

Figure 10. Iron octan, low dose. Iron particles located between the cisternae of the endoplasmic reticulum (**er**) as well as in a vacuole (**v**) of a basophilic cell of the digestive gland.

fed low and high doses of either iron butan or iron octan, or untreated control food, was determined by two filtering electron microscope methods, ESI and EELS. The suitability of these methods to detect iron in biological material postfixed by osmium tetroxide was shown by Stearns *et al*[21] and Triebskorn *et al*.[20] The presence of very weak iron signals in control animals (only detectable in secondary lysosomes in the digestive gland) indicates that the stronger iron signals in treated slugs are attributable to the ingested iron chelate. Although it was not possible with the methods used to distinguish between dissociated iron and that still held in organic complexes, we were able to differentiate between intracellular sites in which iron was located.

The strongest signals for iron were obtained in the large vacuoles/secondary lysosomes of the resorptive cells in the digestive gland of slugs treated with a low dose of either compound, or with the high dose of iron octan. In slugs fed the high dose of iron butan, only weak iron signals were found in these vacuoles, which had a dispersed distribution comparable with those found in controls. The digestive gland is the most important organ of molluscs involved in accumulation and detoxification of metals,[22] and also of organic compounds.[23,24] Our results corroborate the findings of Marigómez *et al*[25] and Triebskorn and Köhler[26] who also detected metals in secondary lysosomes of the resorptive cells in the digestive gland. Récio *et al*[27] and Nott and Nicolaidou,[28] however, found metals to be located in spherites of basophilic

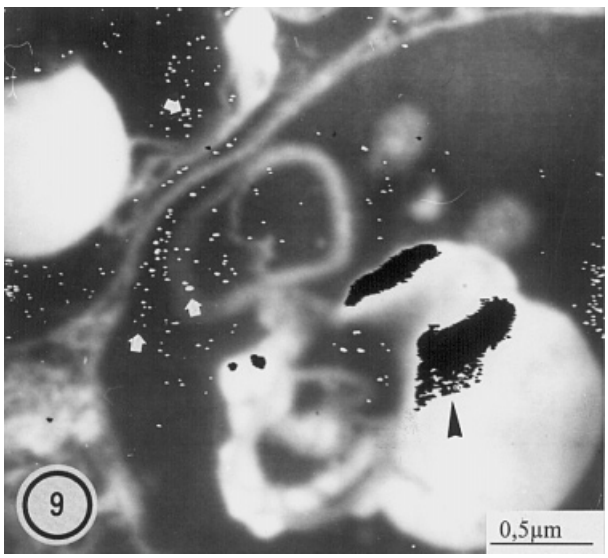

Figure 9. Iron octan, low dose. Strong iron signals attached to electron-dense contents of a secondary lysosome in a resorptive cell of the digestive gland (arrow head), and small, dispersed iron particles surrounding these electron-dense contents (arrows).

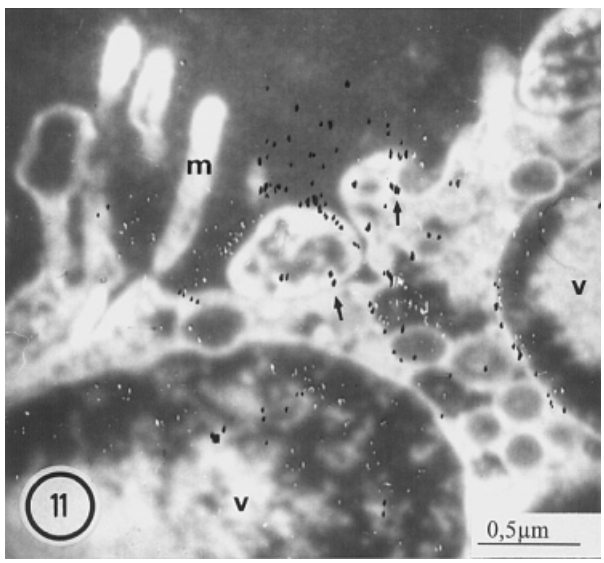

Figure 11. Iron octan, high dose. Iron located in the lumen of the digestive gland tubules between the microvilli (**m**) of a resorptive cell, in the apical cytoplasm of this cell (arrow) as well as in two vacuoles (**v**).

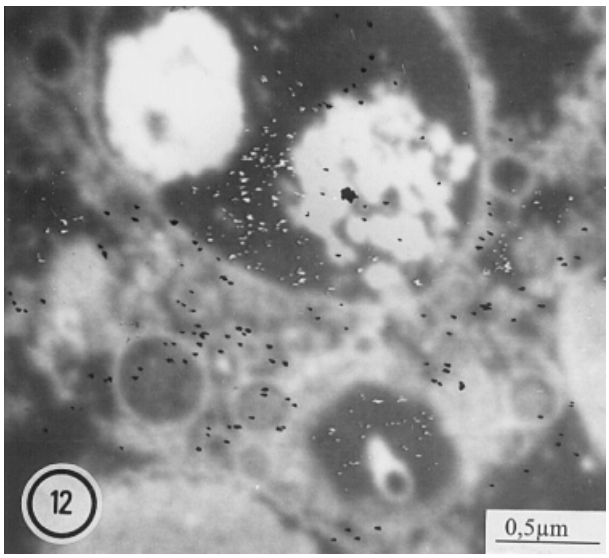

Figure 12. Iron octan, high dose. Iron signals in a secondary lysosome of a resorptive cell in the digestive gland. Iron signals are attached to or surround the electron-dense contents of the vacuole.

cells in this organ. The published data can be interpreted in two ways: (1) for different metals, different ways of uptake might exist; or (2) the secondary lysosomes of the resorptive cells may be an initial, short-term storage site for metals (or certain metals) from which they are released and, in a second step, transferred to the basophilic cells, in which the spherites represent the long-term metal stores. Generally, metal storage and mineralisation is a well-known mechanism in molluscs to cope with potentially high metal concentrations in the environment.[29,30] In no slugs revealing strong iron signals in

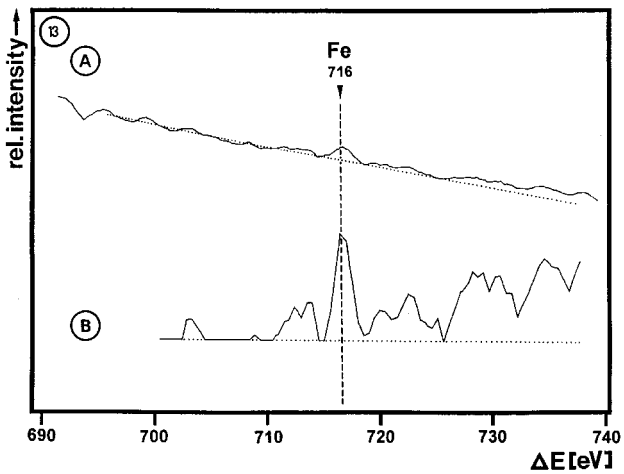

Figure 13. Iron butan, low dose. Electron energy-loss spectrum recorded from a secondary lysosome of a resorptive cell in the digestive gland, where ESI analyses showed the iron to be attached to highly electron-dense contents of the vacuoles (as shown in Figs 2 and 3). The iron signal was found at 716 eV. A: Spectrum originally recorded from the sample. The dotted line represents the extrapolated regression line of the curve. B: The regression line was arbitrarily set to a horizontal baseline. The deviation of the iron peak from this baseline is magnified; signals under the baseline are not shown.

the secondary lysosomes, did the ingested iron chelate exert a toxic effect. It may therefore be possible that the binding of iron in secondary lysosomes of metal-chelate-treated slugs is correlated with a process of detoxification. In addition, iron was found in the lumen of the digestive gland surrounding the microvilli of the resorptive cells, in endocytotic vesicles, and in small vacuoles only in slugs fed with iron octan. This observation corroborates the hypothesis of Clark *et al*[18] that the non-toxic chelate might be better bound to food particles, in this case to paraffin wax in the bait mixture, due to a more lipophilic ligand (the log *P* values for iron butan and iron octan are 1.8 and 5.5 respectively), and might therefore reach the cells of the hepatopancreas via the food pulp. From there, it might have subsequently been resorbed by the cells of the digestive gland. The absence of iron in the apices of midgut gland cells of slugs which had eaten the high dose of iron butan (the toxic chelate) and its presence in epithelial cells and mucocytes of the body wall favour the hypothesis of a quick and effective resorption of iron arising from iron butan in the foregut, followed by its release into the haemolymph. The quick transfer into the haemolymph can be compared with results obtained by Triebskorn *et al*[31] and Triebskorn *et al* (unpublished) for a carbamate molluscicide and for metaldehyde. It corroborates the results of Brooks *et al*[32] who showed that iron fed to *Helix aspersa* (Müller) in barley-flour pellets was absorbed by the crop cells and accumulated in the digestive gland where it could be transported via the haemolymph. A rapid movement of a toxin via the haemolymph to the skin, where it could be excreted via mucus, might be advantageous to the slug. The disadvantage of the haemolymph route, however, might be a rapid distribution of the toxic compound throughout the body and easy access to peripheral organs and to the nervous system. This could result in quicker and more general effects of a potent toxin. For iron butan, such adverse effects seem to far outweigh any possible advantages. Generally, iron chelates are known to cause oxidative damage in target organs and to promote lipid peroxidation.[33,34]

In contrast to slugs fed the high dose of iron butan, in animals treated with the low dose of this compound, strong iron signals were found in secondary lysosomes of the digestive gland, but not at the cell apices of resorptive cells. It seems likely that in slugs fed the low dose of iron butan, iron has entered the digestive gland cells from their bases, as described by Brooks *et al*.[32] In contrast, the iron from iron octan appears to be resorbed by the apices of these cells. As discussed above, these differences in the uptake routes are possibly due to the different properties of the organic residues. High doses of iron butan, however, seem to generally disrupt these processes of iron uptake into the digestive gland. Thus, apart from the chemical properties of the organic ligands, dose-related effects must be considered,

which might only occur after a distinct threshold dose of the toxic compound has reached the respective target sites in the organism. The distribution data of Clark *et al*[18] seem to confirm this; a significant proportion of ingested iron was found in the skin of animals which had eaten high doses of iron butan, whereas only a very small proportion was found in the skin of slugs which had ingested low doses of iron butan or either dose of iron octan. Primary toxic effects could be followed by a series of secondary, indirect effects, e.g. influence on resorption, which in turn, might again have an influence on the toxicity of the test compound. For a carbamate molluscicide, it was shown that the transport of the food pulp via the digestive tract is negatively influenced by the resorption of the compound in the foregut.[35] As a consequence, the food pulp including the toxic compound remained in the foregut, where the resorption of the toxin takes place, for a longer period, thus amplifying the toxic effect. For iron butan, it is possible that the iron from the low dose might be partially resorbed by the cells of the foregut, albeit in very small quantities which might be too low to cause toxic effects. The iron could reach the digestive gland via the haemolymph. In the case of the high dose of iron butan, transport to the digestive gland, via both food pulp and haemolymph, seems to be interrupted. This interruption seems to be correlated with the higher toxicity of this compound applied in high doses.

ACKNOWLEDGEMENTS

The authors are grateful to Dr I Kottke, Botanical Institute, University of Tübingen for providing the filter electron microscope facilities and for discussion, and to Dr H-R Köhler, Zoological Institute, University of Tübingen, for critical reading of the manuscript and his help with the figure layout. IACR-Rothamsted receives grant-aided support from the Biotechnology & Biological Sciences Research Council of the UK.

REFERENCES

1 Byers RB and Calvin DD, *J Econ Entomol* **87**:1345–1350 (1994).
2 Castillejo J, Seijas I and Villoch F, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:327–332 (1996).
3 Adalla CB and Morallo-Rejesus B, in *Slugs and Snails in World Agriculture*, Ed by Henderson, I, BCPC Monograph No. **41**:417–422 (1989).
4 Litsinger JA and Estano DB, *Crop Protection* **12**:363–370 (1993).
5 Arthur JS, Taylor EJ and Bowen ID, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:389–396 (1996).
6 Borlongan IG and Coloso RM, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:205–212 (1996).
7 Martin ID, Mackie GL and Baker MA, *Arch Environ Contam Toxicol* **24**:381–388 (1993).
8 McCullough FS, *Act Trop* **38**:5–13 (1981).
9 Bode AUD, Adewunmi CO, Dörfler G and Becker W, *J Ethnopharmacol* **50**:103–113 (1996).
10 Garthwaite DG and Thomas MR, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:39–46 (1996).
11 Heim IU, Blum RA and Löliger AW, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:47–52 (1996).
12 Marston A and Hostettmann K, Plant molluscicides. *Phytochemistry* **24**:639–652 (1985).
13 Brackenbury T and Appleton CC, *Act Trop* **63**:1–14 (1997).
14 Henderson IF, Briggs GG, Bullock JI, Coward GW, Dawson LF, Larkworthy LF and Pickett, JA, in *Slug and Snails in World Agriculture*, Ed by Henderson, I, BCPC Monograph No. **41**:289–294 (1989).
15 Henderson IF, Martin AP and Parker KA, *Crop Prot* **9**:131–134 (1990).
16 Bullock JI, Coward NP, Dawson GW, Henderson IF, Larkworthy IF, Martin AP and McGrath SP, *Crop Prot* **11**:329–334 (1992).
17 Young C, in *Slug and Snail Pests in Agriculture*, Ed by Henderson, IF, BCPC Monograph No. **66**:237–243 (1996).
18 Clark SJ, Coward NP, Dawson GW, Henderson IF and Martin AP, *Pestic Sci* **44**:381–388 (1995).
19 Spurr AR, *Ultrastr Res* **26**:31–43 (1969).
20 Triebskorn R, Higgins R, Körtje K-H and Storch V, *J Trace Micropr Techn* **14**:775–797 (1996).
21 Stearns RC, Katler M and Godleski JJ, *Microsc Res Tech* **28**:155–163 (1994).
22 Hopkin SP, *Ecophysiology of Metals in Terrestrial Invertebrates*, Elsevier, London. 365 pp (1989).
23 Cajaraville, MP, Uranga JA and Angulo E, *Comp Biochem Physiol* **103**:369–377 (1992).
24 Triebskorn R, *Malacologia* **33**:255–272 (1991).
25 Marigómez I, Soto M and Kortabitate M, *Arch Environ Contam Toxicol* **31**:54–62 (1996).
26 Triebskorn R and Köhler H-R, *Env Poll* **93**:327–343 (1996).
27 Récio A, Marigomez JA, Angulo E and Moja J, *Bull Environ Contamin Toxicol* **41**:858–864 (1988).
28 Nott JA and Nicolaidou A, *Proc R Soc Lond B* 237:347–362 (1989).
29 Berger B and Dallinger R, *Environ Mon Ass* **25**:65–84 (1993).
30 Dallinger R, Strategies of metal detoxification in terrestrial invertebrates, in *Exotoxicology of Metals in Invertebrates*, Ed by Dallinger R and Rainbow PS, Lewis Publishers, Boca Raton, pp 245–289 (1993).
31 Triebskorn R, Künast C, Huber R and Brem G, *Pestic Sci* **28**:321–330 (1990).
32 Brooks AW, White KN and Bailey SE, *Comp Biochem Physiol* **103**:577–583 (1992).
33 Hamazaki S, Okada S, Toyokuni S and Midorikawa O, *Arch Biochem Biophys* **274**:248–254 (1989).
34 Asaumi A, Ogino T, Akiyama T, Kawabata T and Okada S, *Biochem Molec Biol Int* **39**:77–86 (1996).
35 Triebskorn R and Florschütz A, *J Moll Stud* **59**:35–42 (1993).